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(54) Title: PYRODICTIUM XYLANASE, AMYLASE AND PULLULANASE

(57) Abstract

The invention relates to novel thermostable amylases, pullulanases and xylanases obtainable from *Pyrodictium*.

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PYRODICTIUM XYLANASE, AMYLASE AND PULLULANASE

FIELD OF INVENTION

The present invention relates to a novel thermostable amylase and to a novel thermostable pullulanase and their use in the production of sweeteners and ethanol from starch, and to a novel thermostable xylanase and its use in the paper and pulp industry.

BACKGROUND OF THE INVENTION

The production of sweeteners from starch has been largely improved by application of different microbial enzymes to obtain better quality and yields, but the necessity of performing several steps of the starch-hydrolysing process at elevated temperatures means that there is still a need for new starch-hydrolysing enzymes with increased thermal stability.

It is known that Pyrococcus, e.g. Pyrococcus woesei and Pyrococcus furiosus, for reference see Arch. Microbiol. 155, 1991, pp. 572-578, and Appl. Env. Microbiol. 56, 1990, pp.1985-1991, can produce highly thermostable amylases.

The paper and pulp industry is using xylanase compositions in the bleaching process to enhance the brightness of bleached pulps, to decrease the amount of bleaching chemicals, e.g. chlorine, used in the bleaching stages, and to increase the freeness of pulps in the recycled paper process.

Thermostable xylanases from Thermotoga have been described, for reference see Biochem. J. 277(2), 1991, pp. 413-418.

It is the object of this invention to provide a xylanase, an amylase and a pullulanase with temperature optimum at 100°C or above 100°C.

SUMMARY OF THE INVENTION

We have unexpectedly found that a novel thermostable

xylanase, a novel thermostable amylase and a novel thermostable pullulanase can be obtained from the genus Pyrodictium, a genus not previously reported to produce thermostable xylanases, amylases and pullulanases; these new enzymes have temperature optimum around 110-120°C.

Accordingly, the invention provides a xylanase preparation, characterized by being producible by cultivation of a xylanase producing strain of the genus Pyrodictium, and an amylase preparation, characterized by being producible by cultivation of an amylase producing strain of the genus Pyrodictium, and a pullulanase preparation, characterized by being producible by cultivation of a pullulanase producing strain of the genus Pyrodictium.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows the relative activity (% rel.) of an amylase (♦) and a pullulanase (□) of the invention at various temperatures (determined at pH 5.5 with starch and pullulan, respectively, as substrate).

Fig. 2a shows the relative activity (% rel.) of a xylanase of the invention at various temperatures (determined at pH 5.5 with xylan as substrate), and Fig. 2b shows the relative activity of a xylanase of the invention at various pH (determined at 100°C with xylan as substrate).

DETAILED DISCLOSURE OF THE INVENTION

The Microorganism

Looking for extremely thermostable enzymes the extremely thermophilic archaebacteria may be a possible source. Very stable extracellular enzymes from archaebacteria have also been reported, for reference see J.M. Bragger et al. "Very stable enzymes from extremely thermophilic archaebacteria and eubacteria" in Appl. Microbiol. Biotechnol. 31, 1989, p. 556-

561. The genus Pyrodictium, however, has not been reported before to produce extracellular amylases, pullulanases and xylanases, in fact it is the first time a member of the family Desulfurococcaceae has been reported to produce an extracellular xylanase. A survey of the taxonomy of the family Desulfurococcaceae is described in "The Prokaryotes; A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications", 2 nd Ed., Springer-Verlag, Vol I, p. 678.

According to the invention, xylanase is derived from a xylanase producing strain of the family Desulfurococcaceae, in particular the genus Pyrodictium, e.g. P. abyssi, and an amylase is derived from an amylase producing strain of the genus Pyrodictium, in particular P. abyssi, and a pullulanase is derived from a pullulanase producing strain of the genus Pyrodictium, in particular P.abyssi.

A strain representative of Pyrodictium abyssi has been made publicly available under Accession No. DSM 6158. The number is published in the DSM Catalogue of Strains, 1993.

Production of Xylanase, Amylase and Pullulanase

Xylanase, amylase and pullulanase of the invention may be produced by anaerobic cultivation of the above mentioned strain on a nutrient medium containing suitable carbon and nitrogen sources, such media being known in the art. Anaerobic conditions may be achieved during the preparation of media by sparging with H_2/CO_2 (2 bar overpressure) and following the anaerobic techniques as described by Balch and Wolfe in Appl. Env. Microbiol. 32, 1976, pp. 781-791.

Alternatively, xylanase, amylase and pullulanase of the invention can be produced by aerobic cultivation of a transformed host organism containing the appropriate genetic information from the above mentioned strain. Such transformants can be prepared and cultivated by methods known in the art.

The xylanase, amylase and pullulanase may be recovered by removing the cells from the fermentation medium (e.g. by centrifugation or filtration) and then concentrating the

broth (e.g. by ultrafiltration). If desired, the xylanase, amylase and pullulanase may be further purified by known methods.

Immunochemical Properties

The enzymes of the invention have immunochemical properties identical or partially identical (i.e. at least partially identical) to those of an enzyme derived from the strain Pyrodictium abyssi, DSM 6158.

The immunochemical properties can be determined immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to Axelsen N.H.; Handbook of Immunoprecipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), chapters 5 and 14. The terms "antigenic identity" and "partial antigenic identity" are described in the same book, Chapters 5, 19 and 20.

Monospecific antisera are generated according to the above mentioned method by immunizing rabbits with the purified enzymes of the invention. The immunogens are mixed with Freund's adjuvant and injected subcutaneously into rabbits every second week. Antisera are obtained after a total immunization period of 8 weeks, and immunoglobulins are prepared therefrom as described by Axelsen N.H., supra.

The Enzymes

A xylanase of the invention can be characterized by having xylanase activity at temperatures of from below 70°C to approximately 120°C, having activity optimum at temperatures in the range 105-115°C, determined at pH 5.5 with xylan as substrate. The xylanase can also be characterized by having xylanase activity at pH values of from below pH 4.0 to approximately pH 8.5, having optimum in the range pH 5.5 to pH 6.5, determined at 100°C with xylan as substrate.

An amylase of the invention can be characterized by having amylase activity at temperatures of from below 60°C to

above 120°C, having activity optimum at temperatures in the range 110-120°C, determined at pH 5.5 with starch as substrate.

A pullulanase of the invention can be characterized by having pullulanase activity at temperatures of from below 60°C to above 120°C, having activity optimum at temperatures in the range 110-120°C, determined at pH 5.5 with pullulan as substrate.

Determination of Amylase Activity

Amylase activity is determined by measuring the amount of reducing sugar released during the incubation with starch. One unit (U) of amylase activity is defined as the amount of amylase that releases 1 μmole of reducing sugar (as maltose standard) per min. under the following assay conditions: A 0.05 ml volume of 1% soluble starch is added to 0.05 ml of 0.1 M sodium acetate buffer pH 5.5. 25 μl of enzyme solution are added to this mixture and the sample is incubated at 90°C for 30 min. The reaction is stopped by cooling on ice, and the amount of reducing sugar is determined by dinitrosalicylic acid. Sample blanks are used to correct for non-enzymatic release of reducing sugar.

Determination of Pullulanase Activity

Pullulanase activity is determined by measuring the amount of reducing sugar released during the incubation with pullulan. One unit (U) of pullulanase activity is defined as the amount of pullulanase that releases 1 μmole of reducing sugar (as maltose standard) per min. under the following assay conditions: A 0.05 ml volume of 1% pullulan is added to 0.05 ml of 0.1 M sodium acetate buffer pH 5.5. 25 μl of enzyme solution are added to this mixture and the sample is incubated at 90°C for 30 min. The reaction is stopped by cooling on ice, and the amount of reducing sugar is determined by dinitrosalicylic acid. Sample blanks are used to correct for nonenzymatic release of reducing sugar.

Determination of Xylanase Activity

Xylanase activity is determined by measuring the amount of reducing sugar released during the incubation with xylan. One unit (U) of xylanase activity is defined as the amount of xylanase that releases 1 μmole of reducing sugar (as xylose standard) per min. under the following assay conditions: A 0.05 ml volume of 1% soluble xylan is added to 0.05 ml of 0.1 M sodium acetate buffer pH 5.5. 25 μl of enzyme solution are added to this mixture and the sample is incubated at 90°C for 30 min. The reaction is stopped by cooling on ice, and the amount of reducing sugar is determined by dinitrosalicylic acid. Sample blanks are used to correct for nonenzymatic release of reducing sugar.

Industrial Applications

The enzymes of this invention possess valuable properties allowing for various industrial applications. In particular the amylase and pullulanase, in being thermostable, find potential application in the production of sweeteners and ethanol from starch. Conditions for conventional starch converting processes and liquefaction and/or saccharification processes are described in for instance US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909.

Due to the excellent temperature stability of the xylanase, the xylanase of the invention finds potential application in the paper and pulp industry. Use of xylanase in the paper and pulp industry is disclosed in e.g. WO 93/19171.

The following example further illustrates the present invention, and it is not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Xylanase, Amylase and Pullulanase

The strain Pyrodictium abyssi, DSM 6158, was re-cultured from glycerol-preserved cells using the medium recommended by the Deutsche Sammlung von Mikroorganismen (DSM).

The microorganisms were grown in 1 liter of batch cultures under the following conditions: Medium: DSM283 without citric acid (DSM283 is described in DSM Catalogue of Strains, 1993), plus 0.5 g/l of yeast extract plus 0.1 mg/l of $Na_2WO_4X2H_2O$ plus 0.5% (w/v) starch (amylase, pullulanase) or 0.5% (w/v) xylan (xylanase); pH 5.5-6.0; temp. 97°C. The cell density achieved in this medium was ≥ 10^7 cells/ml. Anaerobic conditions were achieved during the preparation of media by sparging with H_2/CO_2 (2 bar overpressure) and following the techniques as described by Balch in Appl. Env. Microbiol. 32, 1976, pp. 781-791.

After cultivation the culture fluid was centrifuged at 12.000 x g for 30 min. at 4°C, and the cell free supernatant was concentrated up to 100-fold using an Amicon Ultrafiltration System.

The following total activity (U) in supernatant was found:

Amylase activity:	2.2 U/l
Pullulanase activity:	3.0 U/l
Xylanase:	1.5 U/l

Temperature Optima

Temperature optima were determined by incubation of samples for 30 minutes at pH 5.5 at temperatures from 60°C to 120°C. The incubation was conducted in closed Hungate tubes in order to prevent boiling of the solution.

Fig. 1 (Amylase (♦) and pullulanase (□)) and Fig 2a (xylanase) show the result.

pH Optima

To determine pH optima of the xylanase, Universal buffer (Britten and Robinson) was used to obtain values from pH 4.0 to pH 8.5. Samples were incubated for 30 minutes at 100°C at the pH in question.

Fig. 2.b shows the result.

CLAIMS

1. A xylanase preparation, characterized by being producible by cultivation of a xylanase producing strain of the family Desulfurococcaceae.

2. A xylanase preparation according to claim 1, wherein said xylanase producing strain belongs to the genus Pyrodictium.

3. A xylanase preparation according to claim 2, wherein said xylanase producing strain belongs to Pyrodictium abyssi.

4. A xylanase preparation according to claim 3, wherein said xylanase producing strain is Pyrodictium abyssi, DSM 6158.

5. A xylanase according to claim 4, further characterized by:

(a) Activity optimum in the range pH 5.5 to pH 6.5, determined at 100°C with xylan as substrate;

(b) Activity optimum at temperatures in the range 105-115°C, determined at pH 5.5 with xylan as substrate.

6. An amylase preparation, characterized by being producible by cultivation of an amylase producing strain of the genus Pyrodictium.

7. An amylase preparation according to claim 6, wherein said amylase producing strain belongs to Pyrodictium abyssi.

8. An amylase preparation according to claim 7, wherein said amylase producing strain is Pyrodictium abyssi, DSM 6158.

9. An amylase according to claim 8, further characterized by activity optimum at temperatures in the range 110-120°C, determined at pH 5.5 with starch as substrate.

10. A pullulanase preparation, characterized by being producible by cultivation of a pullulanase producing strain of the genus Pyrodictium.

11. A pullulanase preparation according to claim 10, wherein said pullulanase producing strain belongs to Pyrodictium abyssi.

12. A pullulanase preparation according to claim 11, wherein said pullulanase producing strain is Pyrodictium abyssi, DSM 6158.

13. A pullulanase preparation according to claim 12, further characterized by activity optimum at temperatures in the range 110-120°C, determined at pH 5.5 with pullulan as substrate.

14. The use of the xylanase according to any of claims 1-5 in a process for treatment of lignocellulosic pulp.

15. The use of the amylase according to any of claims 6-9 in a process of producing sweeteners from starch.

16. The use of the amylase according to any of claims 6-9 in a process of producing ethanol from starch.

17. The use of the pullulanase according to any of claims 10-13 in a process of producing sweeteners from starch.

18. The use of the pullulanase according to any of claims 10-13 in a process of producing ethanol from starch.

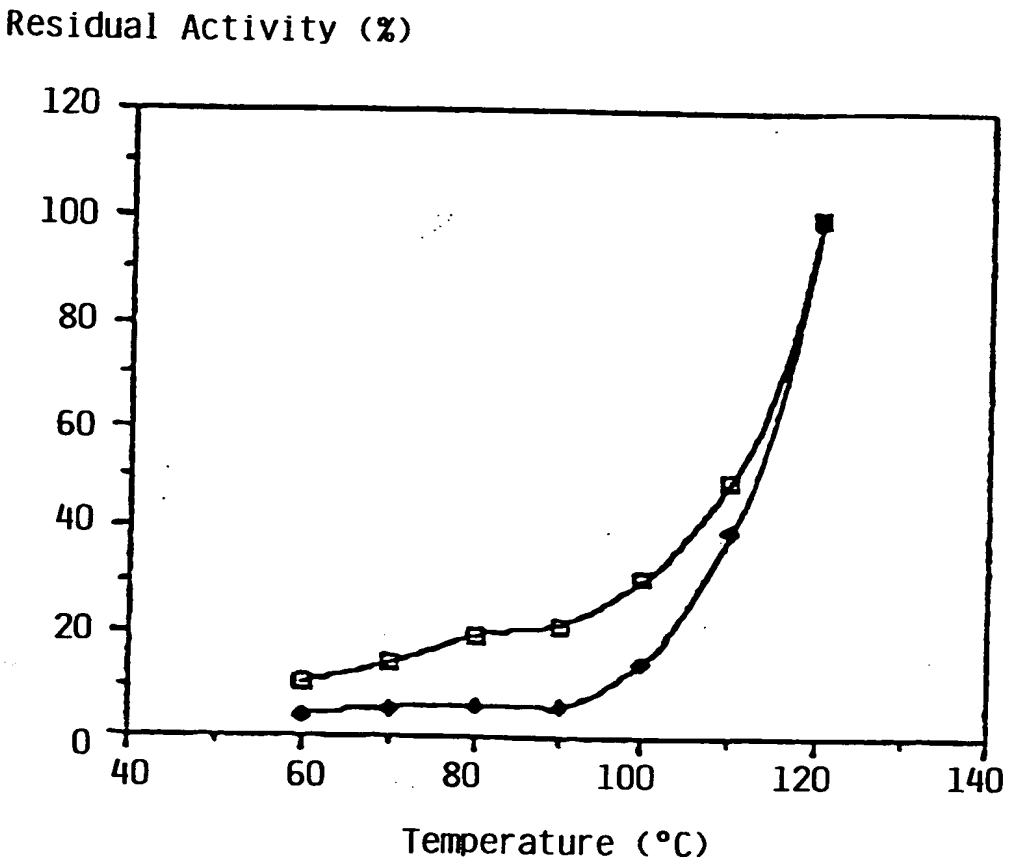
Residual Activity (%)
120
100
80
60
40
20
0
40
60
80
100
120
140
Temperature (°C)

Fig. 1

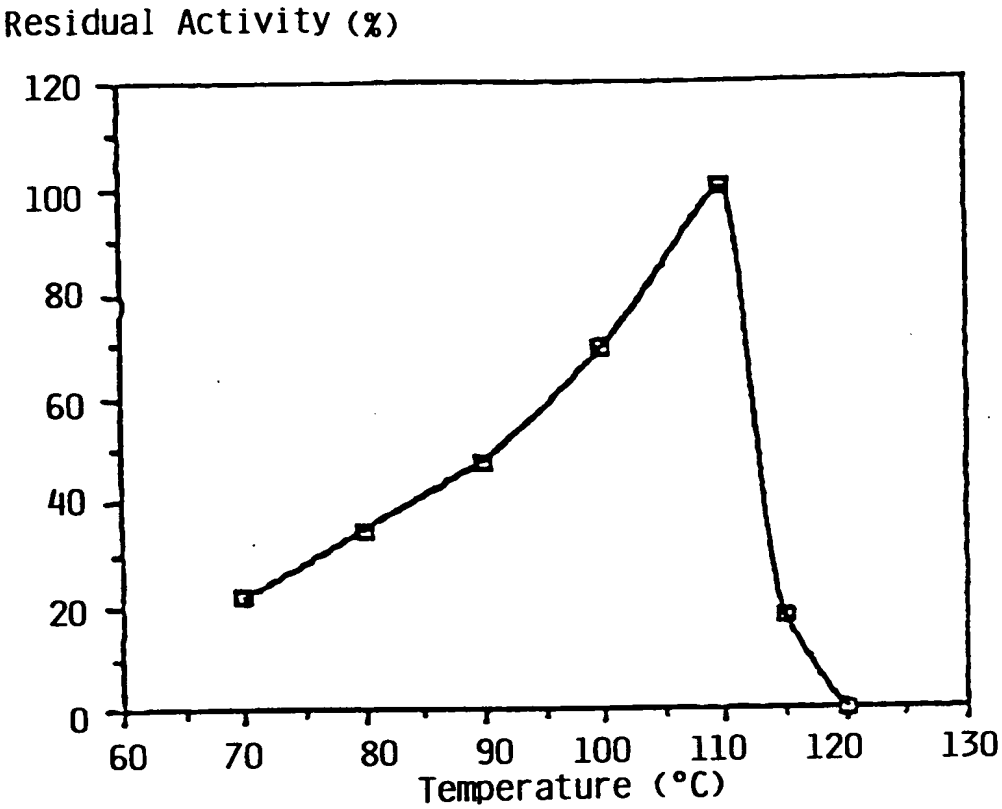
Residual Activity (%)
120
100
80
60
40
20
0
60
70
80
90
100
110
120
130
Temperature (°C)

Fig. 2a

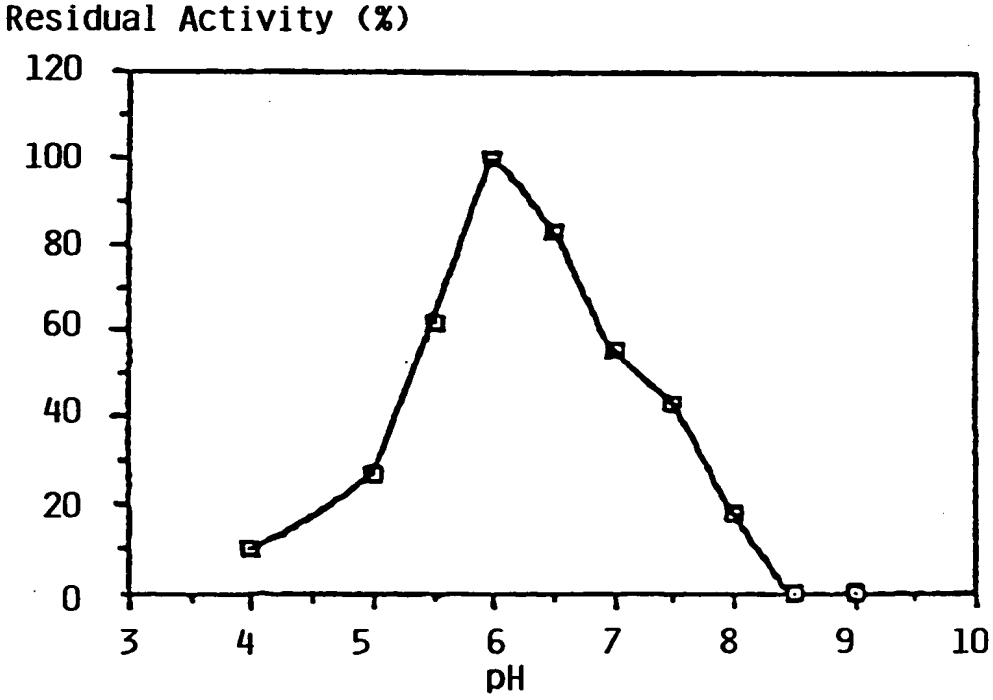
Residual Activity (%)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
pH

Fig. 2b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00211

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/42, C12N 9/24 // C12S 3/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9308275 A1 (NOVO NORDISK A/S), 29 April 1993 (29.04.93), page 5, line 4 - line 8	1-5,14
	--	
X	Chemical Abstracts, Volume 115, No 11, 26 Sept 1991 (26.09.91), (Columbus, Ohio, USA), Simpson, Helen D. et al, "An extremely thermostable xylanase from the thermophilic eubacterium Thermotoga", page 390, THE ABSTRACT No 109212q, Biochem J. 1991, 277 (2), 413-417	1-5,14
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X	WO 9319171 A1 (NOVO NORDISK A/S), 30 Sept 1993 (30.09.93), page 3, line 3 - line 11	1-5,14
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 1995	30 -10- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00211

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4966850 A (ERNEST K.C. YU ET AL), 30 October 1990 (30.10.90), column 4, line 25 - line 30 --	1-5,14
A	Chemical Abstracts, Volume 94, No 21, 25 May 1981 (25.05.81), (Columbus, Ohio, USA), Yoshioka Hajime et al, "Production and characterization of thermostable xylanase from Talaromyces byssochlamydoides", page 298, THE ABSTRACT No 170065h, Agric. Biol. Chem. 1981, 45 (3), 579-586 -- --------	1-5,14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00211

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 1-4 and 14 are too broad and do not disclose the invention in a sufficiently concise manner - see Article 6. The Enzyme is not enough characterized. The indefinate expression "being producible" is not combined with parameters which complete the definition of the invention.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00211

As is stated in Annex B to Administrative Instructions under the PCT, in force July 1, 1992 (PCT GAZETTE 1992, June 25, pages 7062-9, see page 7063 and example 5) unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features"-i.e features that define a contribution which each of the inventions makes over the prior art.

A search for this "special technical feature" mentioned in PCT Rule 13.2 among the independent claims did not reveal such a unifying, novel technical feature. Accordingly, the following inventions were found.

I Claims 1-5 and 14 directed to a thermostable xylanase preparation being producible from Pyrodictium abyssi and its use

II Claims 6-9 and 15-16 directed to a thermostable amylase preparation being producible from Pyrodictium abyssi and its use

III Claims 10-13 and 17-18 directed to a thermostable pullulanase preparation from Pyrodictium abyssi and its use

The inventions claimed are three different enzymes. The three groups of inventions do not form a single general inventive concept.

The search has been restricted to the first invention.

INTERNATIONAL SEARCH REPORT

02/10/95

International application No.
PCT/DK 95/00211

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9308275	29/04/93	EP-A- 0538177	21/04/93
		EP-A- 0610371	17/08/94
		FI-A,D- 941760	15/04/94
		JP-T- 7500730	26/01/95
		NO-A,D- 941379	15/04/94
		NZ-A- 244764	26/07/94
		US-A- 5395765	07/03/95
WO-A1- 9319171	30/09/93	EP-A- 0631621	04/01/95
		JP-T- 7504819	01/06/95
US-A- 4966850	30/10/90	NONE	